DECLARATION

I, the below-named translator, hereby declare:

(1) That my name, mailing address and citizenship are as stated below;

(2) That I am knowledgeable in the English language and in the Korean language in which Korean Patent Application No. 10-2003-0079482 was filed on November 11, 2003; and

(3) That I have translated said Korean Patent Application No. 10-2003-0079482 into English, which English text is attached hereto, and believe that said translation is a true and complete translation of the aforementioned Korean patent application.

February 26, 2008

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KOREAN INTELLECTUAL PROPERTY OFFICE

This is to certify that the following application annexed hereto is a true copy from the records of the Korean Industrial Property Office.

Application Number: 10-2003-0079482

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Applicant(s): National Cancer Center

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COMMISSIONER (seal)

FILING DETAILS OF THE PATENT APPLICATION

Application Number: 10-2003-0079482
Date of Application: November 11, 2003

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[TITLE OF THE INVENTION]
NEUTRALIZABLE EPITOPE OF HGF AND NEUTRALIZING ANTIBODY BINDING TO SAME
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The above application is filed in accordance with Article 42 of Korean Patent Law, and the request for the examination of the above application is filed in accordance with Article 60 of Korean Patent Law.

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ABSTRACT

The present invention relates to a neutralizable epitope of HGF (hepatocyte growth factor) inhibiting the binding of HGF to a receptor thereof and a neutralizing antibody against HGF which is capable of neutralizing HGF as a single agent by binding to said neutralizable epitope of HGF, and provides a neutralizable epitope of HGF having the amino acid sequence of SEQ ID NO: 32 or 33 and a neutralizing antibody against HGF which is capable of neutralizing HGF as a single agent by binding to said neutralizable epitope. The neutralizing antibody neutralizing HGF as a single agent can be effectively used for preventing and treating diseases, specifically cancers caused by binding of HGF to its receptor Met.

REPRESENTATIVE FIGURE

Fig. 9

KEY WORDS

HGF, SF, epitope, neutralizing antibody

SPECIFICATION

TITLE OF THE INVENTION

NEUTRALIZABLE EPITOPE OF HGF AND NEUTRALIZING ANTIBODY BINDING TO SAME

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: the structure of HGF,

Fig. 2: the genetic map of phagemid vector pComb3X used for antibody library construction,

A: the case of displaying Fab on the surface of phagemid, and

B: the case of displaying scFv or diabody on the surface of phagemid

Fig. 3: enrichment of the phage pool displaying Fab specifically binding to HGF through the panning during the culture of HGF-binding clones,

Fig. 4: the result of staining the purified Fab fragments with coomasie blue,

1: marker,

2: non-reduced clone 68 antibody (50,000 Da), and

3: reduced clone 68 antibody (25,000 Da)

Fig. 5: the result of western blotting analysis to determine whether the purified Fab fragments are expressed,

Fig. 6: the binding level of a phage containing the inventive neutralizable epitope to c-MET,

1: phage containing the peptide of SEQ ID NO: 32,

2: phage containing the peptide of SEQ ID NO: 33, and

3 and 4: control phages which do not contain the peptide of SEQ ID NO: 32 or 33

Fig. 7: the specific binding of clone 61 and 68 Fabs to HGF, respectively,

Fig. 8: conformation dependency of the inventive neutralizable epitope defined by clones 61 and 68, respectively,

A: clone 61,

B: clone 68,

Lane 1: non-reduced HGF, and

Lane 2: reduced HGF

Fig. 9B: the criteria showing the cell scattering level ranging from Grades 1 to 6,

Fig. 9A: the result of scattering assay showing that the scattering levels of anti-HGF Fab and anti-human Fab antibodies change with the concentrations of HGF added,

Fig. 10: the amount of clone 68 antibody bound to HGF immobilized on CM5 sensor chip increases with the injected amount of clone 68 antibody,

I: injection of non-specific Fab,

II: injection of 50 nM clone 68 antibody,

III: injection of 100 nM clone 68 antibody,

IV: injection of 200 nM clone 68 antibody,

V: injection of 400 nM clone 68 antibody, and

VI: injection of 600 nM clone 68 antibody

Fig. 11: clone 68 antibody inhibits the binding of HGF to c-Met,

I: injection of 50 nM HGF,

II: injection of 50 nM HGF mixed with 50 nM clone 68 antibody,

III: injection of 50 nM HGF mixed with 250 nM clone 68 antibody,

IV: injection of 50 nM HGF mixed with 500 nM clone 68 antibody,

V: injection of 50 nM HGF mixed with 1 μM clone 68 antibody, and

VI: injection of 50 nM HGF mixed with 1.5 μM clone 68 antibody

Fig. 12: soluble c-Met inhibits the binding of HGF to c-Met.

I: injection of 50 nM HGF,

II: injection of 50 nM HGF mixed with 50 nM soluble c-Met,

III: injection of 50 nM HGF mixed with 100 nM soluble c-Met,

IV: injection of 50 nM HGF mixed with 200 nM soluble c-Met,

V: injection of 50 nM HGF mixed with 400 nM soluble c-Met, and

VI: injection of 50 nM HGF mixed with 600 nM soluble c-Met

DETAILED DESCRIPTION OF THE INVENTION

PURPOSE OF THE INVENTION

FIELD OF THE INVENTION AND PRIOR ARTS

The present invention relates to a neutralizable epitope of HGF and a neutralizing antibody binding to the same, and specifically, relates to a novel neutralizable epitope of HGF inhibiting the binding of HGF to a receptor thereof and a neutralizing antibody which is capable of neutralizing HGF as a single agent by binding to said neutralizable epitope.

HGF (hepatocyte growth factor or SF (scatter factor)) is a multifunctional heterodimeric polypeptide produced by mesenchymal cells.

HGF is composed of an alpha-chain containing an N-terminal domain and four kringle domains (NK4) covalently linked to a serine protease-like beta-chain C-terminal domain (see Fig. 1). Human HGF is synthesized as a biologically inactive single chain precursor consisting of 728 amino acids, and biologically active HGF is achieved through cleavage at the R494 residue by a specific, extracellular serum serine protease. The active HGF thus achieved is a fully active heterodimer which is composed of disulfide linked 69 kDa alpha-chain and 34 kDa beta-chain. However, the overall tertiary structure of HGF is still unknown and it has not yet been clarified which of these domains is responsible for the specific functions of HGF (Maulik et al., *Cytokine & Growth Factor Reviews* 13(1): 1-59, 2002).

The binding of HGF to its receptor, Met, induces the growth and scattering of various cell types, mediates the epithelial mesenchymal transitions and the formation of tubules and lumens, and promotes angiogenesis. Both Met and HGF knockout mice are embryonic lethal and show developmental defects in placenta, fetal liver and limb/muscle formation (Cao et al., *PNAS* 98(13): 7443-7448, 2001; Gmyrek et al., *American Journal of Pathology* 159(2): 579-590, 2001).

Met was originally isolated as a product of a human oncogene, *trp-met*, which encodes a constitutively active altered protein kinase with transforming activity. Met activation has also been shown to remarkably enhance the metastastic spread of cancer stemming from its stimulatory influence of processes such as angiogenesis, cell motility, and cell surface protease regulation (Wielenga et al., *American Journal of Pathology* 157(5): 1563-1573, 2000). Since Met was reported to be over-expressed in various human cancers of liver, prostate, colon, breast, brain and skin (Maulik et al, *supra*), it has been regarded as an important target factor for the prevention and treatment of cancer.

Meanwhile, selective neutralization of tumor-associated growth factors

or cytokines and their receptors, which play crucial roles in the development and spread of cancer, has always been an attractive strategy for the development of anti-cancer drugs. Recently, numerous therapeutic monoclonal antibodies (mAbs) for these targets, e.g., herceptin, and anti-angiopoietin human mAbs have been developed using recombinant antibody technologies such as phage display of combinational antibody library.

It is well known that polyclonal antibodies against HGF block many of HGF biological functions. In addition, it has been recently reported that mixtures of neutralizing mAbs against HGF display anti-tumor activity in animal models (Cao et al., *PNAS* 198(13): 7443-7448, 2001). In particular, Cao et al. disclosed that three or more of the epitopes, possibly two for the Met receptor and one for heparin, need to be blocked in order to inhibit HGF activity *in vivo* and *in vitro*, and a mixture of at least 3 mAbs is capable of neutralizing HGF in an *in vitro* experiment.

However, there has been reported no monoclonal antibody that can neutralize HGF as a single agent and inhibit cell scattering activity *in vitro*.

TECHNICAL OBJECT OF THE INVENTION

It is an object of the present invention to provide a neutralizable epitope of HGF which inhibits the binding of HGF to a receptor thereof.

It is another object of the present invention to provide a neutralizing antibody against HGF which is capable of neutralizing HGF as a single agent by binding to said neutralizable epitope.

CONSTITUTION AND FUNCTION OF THE INVENTION

In accordance with one aspect of the present invention, the present invention provides a neutralizable epitope of HGF having the amino acid sequence of SEQ ID NO: 32 or 33.

In accordance with another aspect of the present invention, the present invention provides a neutralizing antibody binding to said neutralizable epitope.

The present invention is described in detail as follows.

The present invention provides a neutralizable epitope of HGF having the amino acid sequence of SEQ ID NO: 32 or 33.

In order to prepare the neutralizable epitope of HGF in accordance with the present invention, an ELISA study is conducted to examine whether antisera from the immunized rabbits with HGF bind to a recombinant human HGF; and the study has shown that antisera from the HGF immunized rabbits specifically bind to HGF. Then, total RNA is extracted from the HGF immunized rabbits and subjected to cDNA synthesis.

To amplify the variable region comprising rabbit light chain (V_L) (V_κ, V_λ) and heavy chain (V_H) and the constant region comprising human C_κ and C_{H1}, PCRs are performed by using primer combinations of SEQ ID NOs: 1 to 20, and then, light and heavy chains of rabbit/human chimeric antibody are amplified by using the PCR products obtained above as templates. After the amplified rabbit V_L and V_H sequences are combined with the amplified human C_κ and C_{H1} sequences, final PCR products encoding a library of antibody fragments (Fab) are cloned into a vector, and the resulting vector is transformed into a *E. coli* cell to construct a chimeric rabbit/human Fab library. The vector and *E. coli* cell employable in the present invention include all vectors and *E. coli* strains conventionally used in the art without limit, but it is preferable to use phagemid vector pComb3X (the Scripps Research Institute, CA, USA) as an vector and *E. Coli* ER2537 (NEB) as a *E. coli* strain.

Phage clones containing anti-HGF Fab are selected by EIA using HGF-coated ELISA plates and anti-human goat Fab polyclonal antibodies. Phage clones selected above are designated H61 (clone 61) and H68 (clone 68).

H61 and H68 clones are subjected to nucleotide sequencing and their amino acid sequences are determined from the analyzed nucleotide sequences, respectively. In a preferred embodiment of the present invention, nucleotide sequencing is performed according to the dye-labeled primer sequencing method (Chung et al., *J. Cancer Res. Clin. Oncol.* 128: 641-649, 2002). As a result, it has been found that H61 clone is composed of V_H and V_L regions having the nucleotide sequences of SEQ ID NOs: 23 and 24, respectively; and H68 clone comprises V_H and V_L regions having the nucleotide sequences of SEQ ID NOs: 25 and 26, respectively.

The amino acid sequences of the respective V_H and V_L regions of H61

and H68 clones from the analyzed nucleotide sequences suggest that H61 clone is composed of V_H region having the amino acid sequence of SEQ ID NO: 27 and V_L region having the amino acid sequence of SEQ ID NO: 28; and H68 clone, V_H region having the amino acid sequence of SEQ ID NO: 29 and V_L region having the amino acid sequence of SEQ ID NO: 30.

Analysis of the framework region (FR) and complementarity determining region (CDR) in the amino acid sequences of H61 and H68 clones has shown that each of V_H and V_L regions of H61 and H68 clones has 4 FRs and 3 CDRs (see Table 2).

To define a neutralizable epitope of HGF, phage clones showing the binding affinity to anti-HGF H61 and H68 Fabs are selected EIA using anti-HGF H61 Fab- or anti-HGF H68 Fab-coated ELISA plates, phage display of combinatorial peptide library and horseradish peroxidase-conjugated anti-M13 phage goat monoclonal antibodies. In a preferred embodiment of the present invention, the PHD peptide library™ (New England Biolob) is employed as a peptide library.

Selected phage clones are subjected to nucleotide sequencing, and amino acid sequences deduced from the analyzed nucleotide sequences have the amino acid sequences of SEQ ID NOs: 32 and 33, which are found to bind to c-MET (see Fig. 6). These results suggest that an antigen binding site of anti-HGF antibody H61 or H68 mimics a HGF binding site of c-MET and the peptides of SEQ ID NOs: 32 and 33 binding to anti-HGF antibody H61 or H68 mimic a c-MET binding site of HGF. Accordingly, the inventive peptides of SEQ ID NOs: 32 and 33 are capable of functioning as a neutralizable epitope of HGF.

Further, the present invention provides the DNA sequence encoding said neutralizable epitope. In particular, said neutralizable epitope has the DNA sequence of SEQ ID NO: 34 and 35.

Furthermore, the present invention provides a neutralizing antibody against HGF which is capable of neutralizing HGF by binding to the peptide of SEQ ID NO: 32 or 33 as a neutralizable epitope of HGF.

The neutralizing antibody of the present invention may be a chimeric antibody, a monoclonal antibody or a humanized antibody.

The chimeric antibody refers to an antibody whose variable region is derived from a non-human source (e.g. mouse, rabbit, poultry) and the constant region is derived from a human source. In general, the chimeric antibody can

be prepared using one of conventional methods known in the art such as gene recombination method.

A monoclonal antibody refers to an antibody that is produced from hybridoma produced by fusion of B cell producing an antigen specific antibody and myeloma cell which is one of cancer cell strains, and can be prepared using one of conventional methods known in the art.

A humanized antibody refers to an antibody prepared by grafting CDRs (complementarily determining regions) derived from animals to a human antibody in order to maintain the high affinity and specificity of original monoclonal antibody derived from animals, and minimizes immunogenicity in humans

In particular, the present invention provides a rabbit/human chimeric antibody as a neutralizing antibody against HGF. The inventive neutralizing antibody comprises V_H region of SEQ ID NO: 27 and V_L of SEQ ID NO: 28 or V_H region of SEQ ID NO: 29 and V_L of SEQ ID NO: 30.

Whether or not a neutralizing antibody exerts neutralizing activity may be examined by MDCK2 scattering assay (Cao et al., *PNAS* 98(13): 7443-7448, 2001). As a result, in case of treating 2 ng/㎖ of HGF (29 pM) to MDCK2 cells, the inventive neutralizing antibody shows the highest scattering inhibitory activity when the molar ratio of anti-HGF Fab to HGF becomes 50:1, and the molar ratio of anti-human Fab to HGF, ranging from 50:1 to 100:1 (see Fig. 9). These results show for the first time that blocking of only one epitope is sufficient for neutralizing HGF, different from the Cao report that it is necessary to neutralize at least three epitopes to inhibit MDCK2 cell scattering (Cao et al., *supra*). Further, shown in the present invention is the fact that the neutralizing antibody exerts its neutralizing activity only when the antibody binding to the neutralizable epitope is divalent or more, which suggests that the same neutralizable epitope may exist at two or more sites of HGF.

The binding affinity of anti-HGF Fab for HGF, inhibitory activity of clone 68 for binding HGF to c-Met, and inhibitory activity of soluble c-Met for binding HGF to c-Met may also be examined by BIAcore assay. As a result, the amount of clone 68 antibody binding to HGF immobilized on a sensor chip increases with the injection amount of clone 68 antibody (see Fig. 10), and the amount of HGF binding to c-Met decreases as the concentration of clone 68 antibody increases (see Fig. 11). Further, the amount of HGF binding to c-Met immobilized on the sensor chip decreases with increasing the concentration of

soluble c-Met (see Fig. 12).

The above results demonstrate that the inventive neutralizing antibody acts as a single agent which is capable of neutralizing HGF.

Hereinafter, the following Examples are intended to further illustrate the present invention without limiting its scope.

Example 1: HGF immunization and antibody library construction

Over a period of 4 to 5 months, 2 rabbits of the New Zealand White strain were immunized by 5 cutaneous injections of HGF (R&D systems, USA) dispersed in an emulsion of MPL (monophosphoryl lipid A; highly-refined non-toxic lipid A isolated from remutants of *S. minnesota*) + TDM (synthetic trehalose dicorynomycolate; an analogue of trehalose dimycolate from the cord factor of the tubercle bacillus) + CWS (cell wall skeleton; from deproteinized and delipidated cell walls of mycobacteria) adjuvant (Sigma) at 3-week intervals. Antisera from the immunized animals were analyzed for their binding to recombinant human HGF (R&D systems or Research Diagnostics, Inc.) by ELISA using horseradish peroxidase-conjugated anti-rabbit Fc goat polyclonal antibodies (Pierce). As a result, it was found that while antisera obtained before HGF immunization almost never bind to HGF, antisera obtained after 5 cutaneous injections specifically bound to HGF.

Seven days after the final boost, the spleen and bone marrow were extracted from the immunized animals and used for total RNA preparation with TRI reagent (Molecular Research Center, Cincinnati, USA) and lithium chloride precipitation. First-strand cDNA was synthesized using the SUPERSCRIPT Preamlification System with oligo (dT) priming (Life Technologies, Inc.).

Rabbit/human chimeric antibody library was constructed according to the method described by Rader et al (Rader C. et al., *J. Biol. Chem.* 275: 13668-13676, 2000).

Example 2: Amplification of rabbit-derived Ab variable region and human-derived Ab constant region

(2-1) Amplification of rabbit-derived Ab variable region

In order to amplify variable regions of rabbit V_L (V_κ, V_λ) and V_H, PCR

was performed by using primer combinations described in Table 1.

<Table 1>

Variable region	Forward primer	Reverse primer
V_κ	SEQ ID NO: 1	SEQ ID NO: 4
	SEQ ID NO: 1	SEQ ID NO: 5
	SEQ ID NO: 1	SEQ ID NO: 6
	SEQ ID NO: 2	SEQ ID NO: 4
	SEQ ID NO: 2	SEQ ID NO: 5
	SEQ ID NO: 2	SEQ ID NO: 6
	SEQ ID NO: 3	SEQ ID NO: 4
	SEQ ID NO: 3	SEQ ID NO: 5
	SEQ ID NO: 3	SEQ ID NO: 6
V_λ	SEQ ID NO: 7	SEQ ID NO: 8
V_H	SEQ ID NO: 9	SEQ ID NO: 13
	SEQ ID NO: 10	SEQ ID NO: 13
	SEQ ID NO: 11	SEQ ID NO: 13
	SEQ ID NO: 12	SEQ ID NO: 13

A PCR reaction solution was prepared by mixing 1 $\mu\ell$ of template cDNA (about 0.5μg) synthesized in Example 1, 60 pmol of each primer, 10 $\mu\ell$ of 10× PCR buffer, 8$\mu\ell$ of 2.5 mM dNTP mixture and 0.5$\mu\ell$ of Taq polymerase and adjusted to a final volume of 100 $\mu\ell$. The PCR condition was 30 cycles of 15 sec at 94℃, 30 sec at 56℃ and 90 sec at 72℃ after initial denaturation of 10 min at 94℃, and final extension of 10 min at 72℃. The amplified DNA was subjected to agarose gel electrophoresis and purified from the gel by using Qiaex gel extraction kit (Qiagen).

(2-2) Amplification of human-derived Ab constant region

PCR was conducted to amplify C_κ region of human-derived Ab constant region as follows: A PCR reaction solution was prepared by mixing 20 ng of pComb3XTT vector (Barbas et al., *Proc. Natl. Acad. Sci. USA* 15:88(18), 7978-82, 1991), 60 pmol of each primer (SEQ ID NOs: 14 and 15), 10$\mu\ell$ of 10× PCR buffer, 8$\mu\ell$ of 2.5 mM dNTP mixture and 0.5$\mu\ell$ of Taq polymerase and adjusted to a final volume of 100 $\mu\ell$. The PCR condition was 20 cycles of 15 sec at 94℃, 30 sec at 56℃ and 90 sec at 72℃ after initial denaturation of 10

min at 94℃, and final extension of 10 min at 72℃.

Meanwhile, PCR was performed to amplify C_{H1} region of human-derived Ab constant region as follows: A PCR reaction solution was prepared by mixing 20 ng of pComb3XTT vector (Barbas et al., *supra*), 60 pmol of each primer (SEQ ID NOs: 16 and 17), 10㎕ of 10× PCR buffer, 8㎕ of 2.5 mM dNTP mixture and 0.5㎕ of Taq polymerase and adjusted to a final volume of 100 ㎕. The PCR condition was 20 cycles of 15 sec at 94℃, 30 sec at 56℃ and 90 sec at 72℃ after initial denaturation of 10 min at 94℃, and final extension of 10 min at 72℃.

The amplified DNAs were subjected to agarose gel electrophoresis and purified from the gel by using Qiaex gel extraction kit (Qiagen).

Example 3: Amplification of light and heavy chains of chimeric antibody

(3-1) Amplification of light chain

PCR was carried out to amplify the light chain as follows: A PCR reaction solution was prepared by mixing 100 ng each of V_L (V_κ, V_λ) PCR product purified in Example (2-1) and C_κ PCR product purified in Example (2-2), 60 pmol each of primers (SEQ ID NOs: 18 and 15), 10㎕ of 10× PCR buffer, 8㎕ of 2.5 mM dNTP mixture and 0.5㎕ of Taq polymerase and adjusted to a final volume of 100 ㎕. The PCR condition was 20 cycles of 15 sec at 94℃, 30 sec at 56℃ and 120 sec at 72℃ after initial denaturation of 10 min at 94℃, and final extension of 10 min at 72℃.

The amplified DNA was subjected to agarose gel electrophoresis and purified from the gel by using Qiaex gel extraction kit (Qiagen).

(3-2) Amplification of heavy chain

Overlap extension PCR was conducted to amplify Fd region (V_H and C_{H1}) of a heavy chain as follows: A PCR reaction solution was prepared by mixing 100 ng each of V_H PCR product purified in Example (2-1) and C_{H1} PCR product purified in Example (2-2), 60 pmol each of primers (SEQ ID NOs: 19 and 17), 10㎕ of 10× PCR buffer, 8㎕ of 2.5 mM dNTP mixture and 0.5㎕ of Taq polymerase and adjusted to a final volume of 100 ㎕. The PCR condition was 20 cycles of 15 sec at 94℃, 30 sec at 56℃ and 120 sec at 72℃ after initial denaturation of 10 min at 94℃, and final extension of 10 min at

72℃.

The amplified DNA was subjected to agarose gel electrophoresis and purified from the gel by using Qiaex gel extraction kit (Qiagen).

Example 4: Preparation of chimeric Fab library

PCR was carried out to amplify chimeric rabbit/human Fab gene as follows: A PCR reaction solution was prepared by mixing 100 ng each of chimeric light chain product purified in Example (3-1) and the chimeric heavy chain product purified in Example (3-2), 60 pmol each of primers (SEQ ID NOs: 18 and 20), 10㎕ of 10× PCR buffer, 8㎕ of 2.5 mM dNTP mixture and 0.75㎕ of Taq polymerase and adjusted to a final volume of 100 ㎕. The PCR condition was 20 cycles of 15 sec at 94℃, 30 sec at 56℃ and 180 sec at 72℃ after initial denaturation of 10 min at 94℃, and final extension of 10 min at 72℃.

The amplified DNA was subjected to agarose gel electrophoresis and purified from the gel by using Qiaex gel extraction kit (Qiagen).

After the PCR products encoding rabbit V_L and V_H sequences were combined with the PCR products encoding human C_κ and C_{H1} sequences, final PCR fragments encoding a library of antibody fragments were subjected to SfiI digestion, purified and cloned into phagemid vector pComb3X (the Scripps Research Institute, CA, USA) (Fig. 2). Phagemid DNA was transformed into *E. coli* ER2537 (NEB) by electrophoration. The introduced phage displayed Fab as a fusion protein fused on phage coat protein pⅢ and its DNA formed a phage particle (gene and polypeptide as one unit) in the phage DNA.

Example 5: Selection of phage clone containing anti-HGF Fab

After a 96-well plate (Costar No. 3690) was coated with HGF dissolved in 25㎕ of TBS solution at a concentration of 10㎍/㎖ per well, phages displaying Fab prepared in Example 4 were added to the well plate, the well plate was kept at room temperature for 2 hrs, and panned against immobilized HGF antigen at the well plate. The well plate was washed with 0.5% (v/v) Tween 20 in PBS and eluted with 0.1 M HCl-glycine (pH 2.2). The washing steps were increased from 5 times in the first round to 10 times in the second round and 15 times in the following rounds. Typically seven rounds of

panning were conducted. As the panning proceeded, phage pools displaying anti-HGF Fab which specifically bind to HGF increased, which results in increasing absorbance showing the HGF binding to Fab in EIA using HRP-conjugated anti-M13 phage antibodies (Pharmacia) and HGF-coated ELISA plates (Fig. 3). After the last round of panning, the phage clones containing anti-HGF Fab were selected by EIA using the HGF-coated ELISA plates and goat anti-human Fab polyclonal antibodies (Pierce), respectively. The selected clones were designated H61 (clone 61) and H68 (clone 68). H61 and H68 clones which gave specifically strong signals against HGF above background (Fig. 7) were further analyzed by nucleotide sequencing, and the amino acid sequences were determined from the analyzed nucleotide sequences.

Example 6: Nucleotide sequencing analysis of selected phages

Nucleotide sequencing was carried out by the dye labeled primer sequencing method (Chung et al., *J. Cancer Res. Clin. Oncol.* 128: 641-649, 2002) using two sequencing primers of SEQ ID NOs: 21 and 22. As a result, it was found that H61 clone encodes the anti-HGF Fab consisting of V_H region having the nucleotide sequence of SEQ ID NO: 23 and V_L region having the nucleotide sequence of SEQ ID NO: 24; and H68 clone, the anti-HGF Fab consisting of V_H region having the nucleotide sequence of SEQ ID NO: 25 and V_L region having the nucleotide sequence of SEQ ID NO: 26.

The amino acid sequences of H61 and H68 clones were deduced from the analyzed nucleotide sequences, respectively. As a result, it was found that V_H and V_L regions of H61 clone had the amino acid sequences of SEQ ID NO: 27 and 28, respectively, while V_H and V_L regions of H68 clone had the amino acid sequences of SEQ ID NO: 29 and 30, respectively.

As a result of analyzing a framework region (FR) and complementarity determining region (CDR) in the amino acid sequences of H61 and H68 clones according to the method described by Harris (Harris et al., *Protein Science* 4(2): 306-10, 1995), H61 and H68 clones had the regional constituents described in Table 2.

<Table 2>

Region	H61 clone V_H	H61 clone V_L	H68 clone V_H	H68 clone V_L
FR1	1-30	1-23	1-30	1-23
CDR1	31-35	24-34	31-35	24-34
FR2	36-49	35-49	36-49	35-49
CDR2	50-66	50-56	50-66	50-56
FR3	67-98	57-88	67-98	57-88
CDR3	99-105	89-97	99-105	89-97
FR4	106-116	98-109	106-116	98-109

Example 7: Anti-HGF Fab expression and purification for *in vitro* assay

Phagemid DNAs of the selected clones in Example 5 were transformed into non-suppressor *E. coli* strain HB2151. Clones were grown to an A600 nm absorbance of 0.5 to 1.0 and induced the expression of anti-HGF Fab with IPTG (1 mM) for 20 to 24 hrs. Culture supernatants were concentrated by Labscale TFF system (Millipore). Concentrated anti-HGF Fab was purified by affinity chromatography using anti-HA tag mouse monoclonal antibody. Purified Fab fragments were analyzed by Coomassie staining and western blotting.

First, the purified H68 antibody Fab (about 1-3μg) was subjected to electrophoresis by loading on NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen). The loaded gel was soaked in a coomassie gel staining solution (Invitrogen), stirred for 30 min, transferred into a coomassie gel destaining solution, and stirred until developed protein bands were observed. Fig. 4 shows the result of coomassie staining. As shown in Fig. 4, in case of non-reduced H68 antibody (lane 2), most of Fab antibodies were detected at a position corresponding to a molecular weight of 50,000 Da; in case of reduced H68 antibody (lane 3), Fab antibody was separated into the respective Fd regions of light and heavy chains, and therefore, bands were detected at a position corresponding to 25,000 Da; and other bands except antibody bands were not detected. In view of facts that light and heavy chains of H68 antibody Fab are of the size of about 25,000 Da, respectively, and Fab formed by covalently linking between the light and heavy chains by a disulfide bond has the size of about 50,000 Da, the H68 antibody Fab was successively isolated and purified to a satisfiable purity. However, the weak band detected in lane 2 at a position corresponding to 25,000 Da is due to the presence of free Fd regions of Fab light and heavy chains that are not

linked to each other.

Meanwhile, for western blotting, the purified anti-HGF Fab (about 1-3μg) was subjected to electrophoresis by loading on NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen). The Fab separated according to molecular weight was immobilized onto BioTrace Nitrocellulose membrane (PALL). The membrane was treated with 5% non-fat dry milk/TBS for 30 min to block. Horseradish peroxidase-conjugated anti-human goat Fab polyclonal antibody (Pierce) was diluted with 3% non-fat dry milk/TBS in a ratio of 1:1000 and reacted with the membrane for 1 hr with stirring. The membrane was washed with TBS for 30 min and wetted evenly with an equal volume mixture of Supersignal West Pico stable peroxide solution (Pierce) and Supersignal West Pico Luminol/Enhancer solution (Pierce). The membrane was exposed to X-ray film (Kodak) in a darkroom, and the result was shown in Fig. 5.

In Fig. 5, the left lane is a size marker and other lanes are the purified Fabs. As shown in Fig. 5, a large quantity of Fab was detected at a position corresponding to 50,000 Da, and free Fd regions of light and heavy chains, at a position corresponding to 25,000 Da.

Example 8: Analysis of nucleotide sequence and features of HGF neutralizable epitope

(8-1) Nucleotide sequencing

A 96-well plate (costar No. 3690) was coated with anti-HGF H61 Fab or anti-HGF H68 Fab dissolved in 25$\mu\ell$ of TBS solution at a concentration of 10μg/mℓ per well. PHD peptide library™ (phage display of combinatorial peptide library) (New England Biolob.) was added to the well plate, and then, the well plate was kept for 2 hrs at room temperature. The well plate was washed with 0.5% (v/v) Tween 20 in PBS and eluted with 0.1M HCl-glycine (pH 2.2). The washing steps were increased from 5 times in the first round to 10 times in the second round and 15 times in the subsequent rounds. Typically seven rounds of panning were carried out. After the last round of panning, the phage clones containing anti-HGF H61 Fab or anti-HGF H68 Fab were selected by EIA using anti-HGF H61 Fab or anti-HGF H68 Fab-coated ELISA plate and horseradish peroxidase-conjugated anti-M13 phage goat monoclonal antibody (Roche). The selected clones were subjected to nucleotide sequencing and the amino acid sequences were determined from the analyzed nucleotide sequences.

Nucleotide sequencing was performed according to the dye-labeled primer sequencing method (Chung et al., *supra*) using a sequencing primer of SEQ ID NO: 31. As a result, peptides encoding anti-HGF H61 and H68 Fabs deduced from the analyzed nucleotide sequences had the amino acid sequences of SEQ ID NOs: 32 and 33, respectively.

Then, a 96-well plate (costar No. 3690) was coated with c-Met dissolved in 25$\mu\ell$ of TBS solution at a concentration of 10μg/mℓ per well, and clones containing the peptides of SEQ ID NOs: 32 and 33 were each added thereto. The well plate was washed with 0.5% (v/v) Tween 20 in PBS, and horseradish peroxidase-conjugated anti-M13 phage goat monoclonal antibody (Roche) was added thereto.

As shown in Fig. 6, while the phages containing the respective peptides (1 and 2) of SEQ ID NO: 32 and 33 bound to c-Met, two control phages (3 and 4) without said peptides did not. These results suggest that since an antigen binding site of anti-HGF antibody H68 mimics a HGF binding site of c-Met and the peptides of SEQ ID NOs: 32 and 33 binding to clone 68 mimic c-Met binding site of HGF, the peptides of SEQ ID NOs: 32 and 33 can function as a neutralizable epitope of HGF.

(8-2) Characterization

In order to characterize the antigen binding site of anti-HGF antibody, western blotting was carried out as follows. 1 to 3μgof HGF was subjected to electrophoresis by loading on NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen). At this time, some were loaded after treating with a reducing agent and others were loaded without such reducing agent treatment. Proteins separated according to molecular weight were immobilized onto BioTrace Nitrocellulose membrane (PALL). The membrane was each treated with 5% non-fat dry milk/TBS for 30 min to block. Anti-HGF H61 and H68 Fabs were added to the membrane, and the membrane was stirred for 1 hr. Horseradish peroxidase-conjugated anti-human goat Fab polyclonal antibody (Pierce) was diluted with 3% non-fat dry milk/TBS in a ratio of 1:1000 and reacted with the membrane for 1 hr with stirring. The membrane was washed with TBS for 30 min and wetted evenly with an equal volume mixture of Supersignal West Pico stable peroxide solution (Pierce) and Supersignal West Pico Luminol/Enhancer solution (Pierce) for 30 min. The membrane was exposed to X-ray film

(Kodak) in a darkroom, and the result was shown in Fig. 8.

In Fig. 8, an arrow indicates a size marker; A, clone 61; B, clone 68; lane 1, a non-reduced HGF; and lane 2, a reduced HGF. As a result, clones 61 and 68 were found to bind to the non-reduced HGF, but not to the reduced HGF. These results suggest that the tertiary structure of the antigenic determinants, e.g., epitopes, serving as binding sites of clones 61 and 68, are crucial for antigen-antibody response and the inventive neutralizable epitopes has a non-linear structure.

Example 9: MDCK scattering assay

MDCK cells (Madine Darby canine kidney cells; ATCC CCL 34) were cultured in a DMEM medium supplemented with 5% FCS at 37℃ in a humid chamber under 95% air and 5% CO_2. Cells were distributed on a 96-well plate at a concentration of 2×10^3 cells/well and exposed to 2 ng/㎖ (29 pM) of HGF in a fresh medium overnight. Then, anti-HGF Fab and anti-human Fab antibodies were added to the well plate at different concentrations. The scattering effect was monitored by light microscopy, and the result was shown in Fig. 9. Fig. 9B shows the criteria indicating the cell scattering level ranging from Grades 1 to 6, wherein Grade 6 means 100% inhibition of scattering effect by HGF; Grade 5, inhibition ranging from 90 to 100% of scattering effect by HGF; Grade 4, inhibition ranging from 60 to 90% of scattering effect by HGF; Grade 3, inhibition ranging from 30 to 60% of scattering effect by HGF; Grade 2, inhibition ranging from 10 to 30% scattering effect by HGF; and Grade 1, 10% and less inhibition of scattering effect by HGF. Fig. 9A shows that the scattering levels of anti-HGF Fab and anti-human Fab antibodies may differ according to the concentrations of HGF added.

As a result, it was found that the most effective scattering effect was expected when the molar ratio of anti-HGF Fab to HGF was 50:1 and the molar ratio of anti-human Fab antibody to HGF was ranging from 50:1 to 100:1.

Example 10: BIAcore assay

(10-1) Affinity analysis of anti-HGF Fab for HGF

The binding affinity of anti-HGF Fab for HGF was determined by SPR (surface plasmon resonance) using the BIAcore 3000 (BIAcore AB, Uppsala,

Sweden).

Approximately, 1069 resonance units (RU) of HGF were coupled to CM5 sensor chip (BIAcore AB) through an amine coupling method. Binding interaction was allowed to proceed in PBS buffer containing 0.005% surfactant P20 at a flow rate of 30 $\mu\ell$/min at 25℃. The surface was regenerated with 1 M NaCl/50 mM NaOH. The kinetic rate constants (k_{on} and k_{off}) as well as the equilibrium dissociation constant (K_d) were determined. Fig. 10 shows the binding affinity of anti-HGF H68 Fab for HGF. As a result, it was found that the amount of anti-HGF H68 Fab bound to HGF immobilized on the sensor chip increases with the concentration of anti-HGF H68 Fab.

(10-2) Analysis of HGF binding inhibitory activity of clone 68 antibody against HGF

To ascertain the fact that anti-HGF H68 Fab can inhibit the binding of HGF to c-Met in real time, c-Met was coupled to CM5 sensor chip through an amine coupling method. Thereafter, HGF alone was injected at a concentration of 50 nM, and premixed with anti-HGF H68 Fab of 5 different concentrations (50 nM, 250 nM, 500 nM, 1 μM and 1.5 μM) and soluble c-Met of 5 different concentrations (50 nM, 100 nM, 200 nM, 400 nM, 600 nM), respectively. Binding interaction was allowed to proceed in PBS buffer containing 0.005% surfactant P20 at a flow rate of 30 $\mu\ell$/min at 25℃. The surface was regenerated with 1 M NaCl/50 mM NaOH.

Fig. 11 shows that anti-HGF H68 Fab inhibits the binding of HGF to c-Met. As a result, in case of 50 nM HGF injection, HGF was found to bind to c-Met at 455.5 RU (Ⅰ), while in case of 50 nM HGF injection with anti-HGF H68 Fab at 5 different concentrations of 50 nM (Ⅱ), 250 nM (Ⅲ), 500 nM (Ⅳ), 1 μM (Ⅴ) and 1.5 μM (Ⅵ), HGF bound to c-Met at 406.5, 328, 260, 111.1 and 71 RU, respectively. These results suggest that the binding of HGF to c-Met becomes reduced as the concentrations of anti-HGF H68 Fab increases. There was no binding of HGF in case when anti-HGF H68 Fab alone was injected.

(10-3) Analysis of HGF binding inhibitory activity of soluble c-Met against c-Met

Whether the binding of HGF to c-Met is inhibited by soluble c-Met was

examined as follows. 2979 RU of c-Met was immobilized on CM5 sensor chip through an anime coupling method. Binding interaction was allowed to proceed in PBS buffer containing 0.005% surfactant P20 at a flow rate of 30 $\mu\ell$/min at 25℃. The surface was regenerated with 1 M NaCl/50 mM NaOH, and the result was shown in Fig. 12.

As shown in Fig. 12, in case of 50 nM HGF injection, HGF was found to bind to c-Met at 455.5 RU (Ⅰ); while in case of 50 nM HGF injection with soluble c-Met at 5 different concentrations of 50 nM (Ⅱ), 100 nM (Ⅲ), 200 nM (Ⅳ), 400 nM (Ⅴ) and 600 nM (Ⅵ), HGF bound to c-Met at 310.3, 225.7, 167.4, 93.7 and 70.9 RU, respectively. These results suggest that the amount of HGF binding to c-Met immobilized on the sensor chip gradually decreases with increasing concentration of soluble c-Met increases.

Effect of the Invention

The neutralizing antibody which is capable of neutralizing HGF as a single agent by binding to the neutralizable epitope of HGF of the present invention can be effectively used for preventing and treating diseases, specifically cancers caused by binding of HGF to its receptor Met.

WHAT IS CLAIMED IS:

1. A neutralizable epitope of HGF (hepatocyte growth factor) having the amino acid sequence of SEQ ID NO: 32 or 33.

2. A DNA encoding the neutralizable epitope of claim 1.

3. The DNA of claim 2, which has the nucleotide sequence of SEQ ID NO: 34 or SEQ ID NO: 35.

4. A neutralizing antibody which binds to the neutralizable epitope of claim 1.

5. The neutralizing antibody of claim 4, which is selected from the group consisting of a chimeric antibody, a monoclonal antibody and a humanized antibody.

6. The neutralizing antibody of claim 4, which comprises V_H region having the amino acid sequence of SEQ ID NO: 27 and V_L region having the amino acid sequence of SEQ ID NO: 28.

7. The neutralizing antibody of claim 4, which comprises V_H region having the amino acid sequence of SEQ ID NO: 29 and V_L region having the amino acid sequence of SEQ ID NO: 30.

FIGURE

Fig. 1

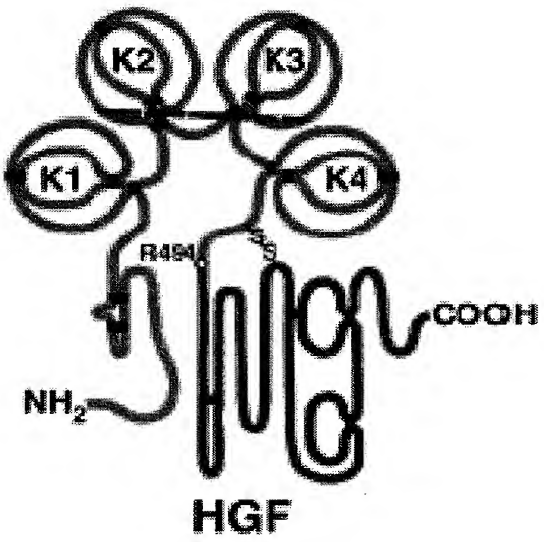

Fig. 2

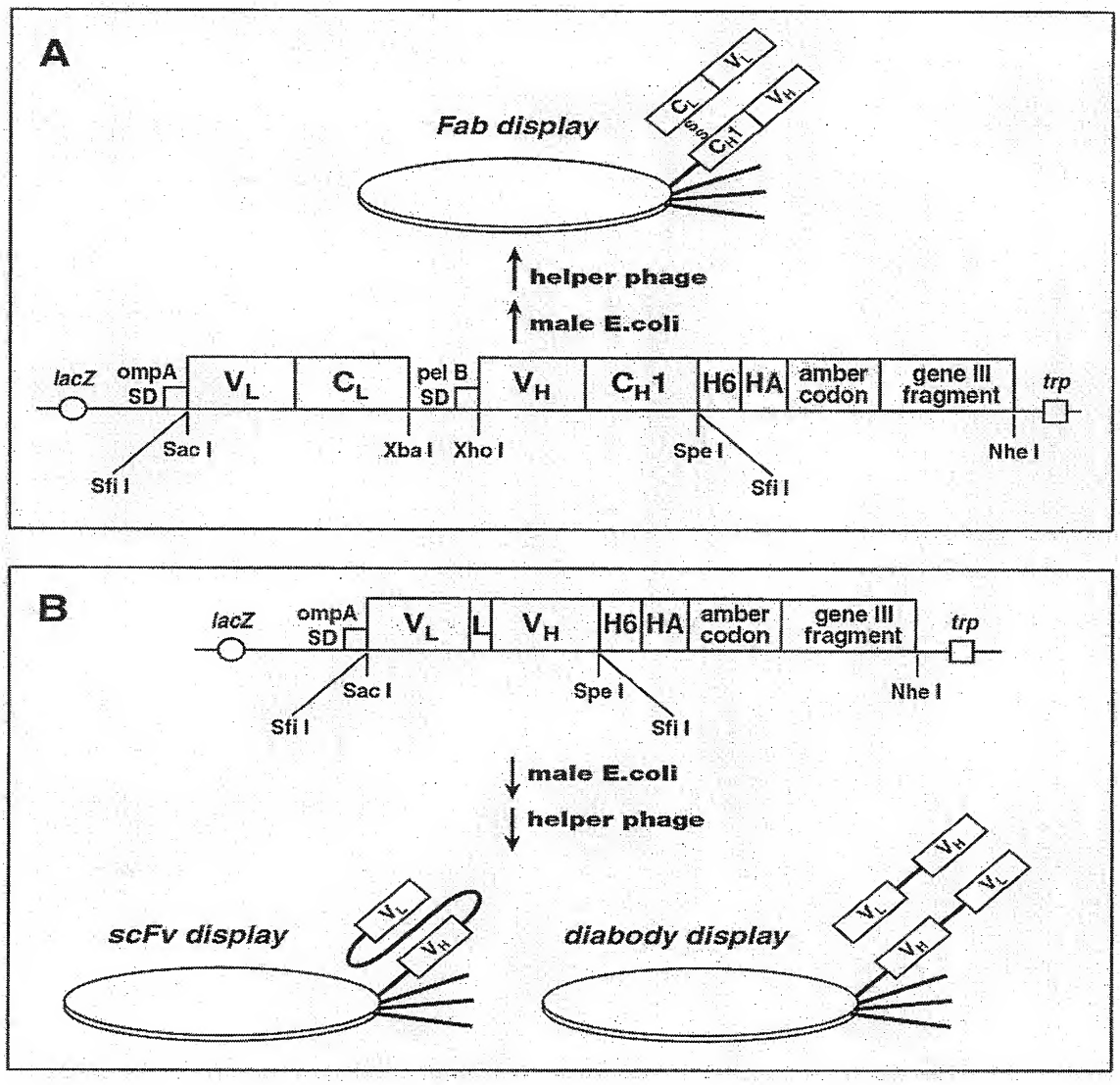
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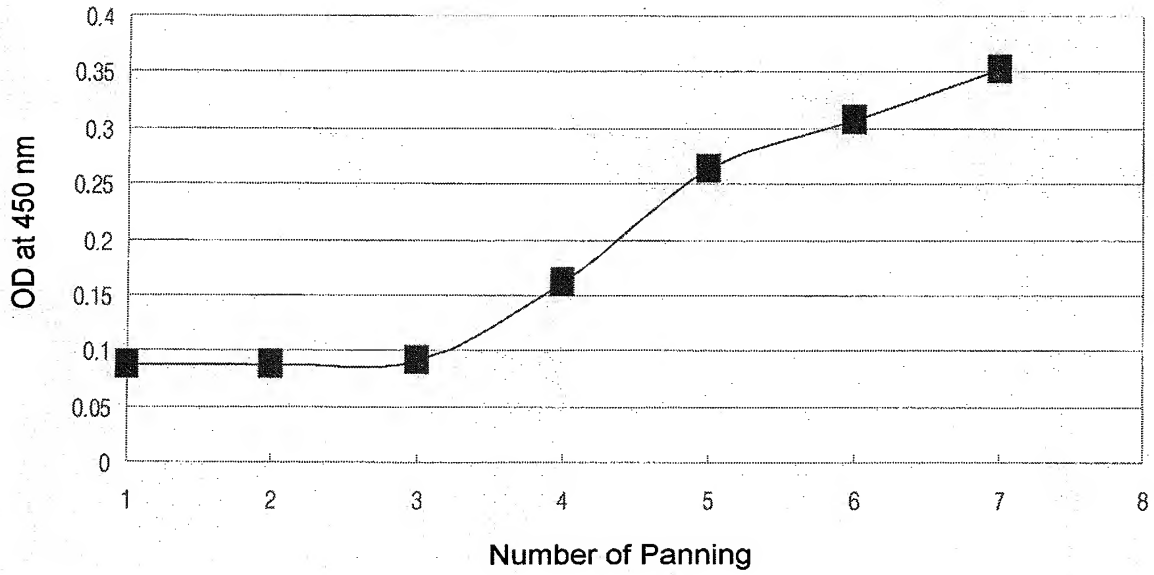
Fig. 3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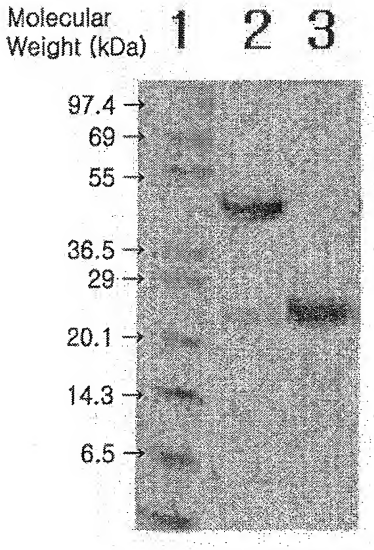
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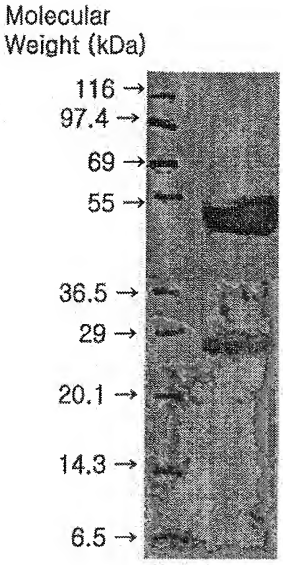
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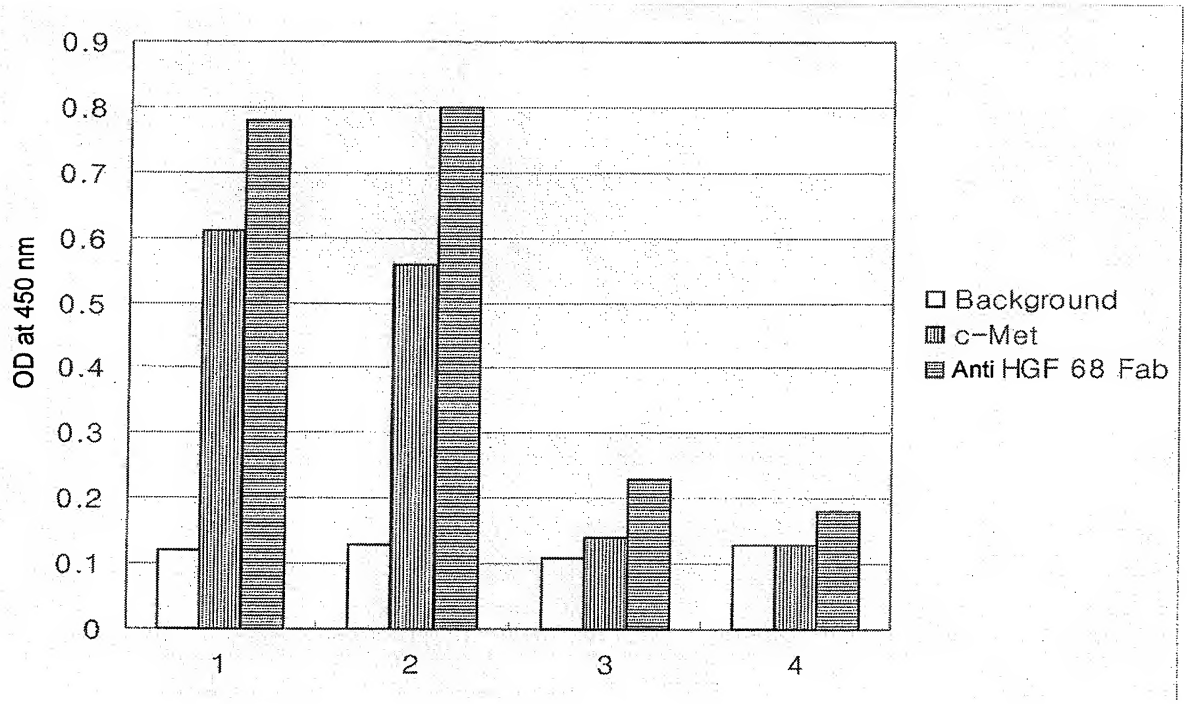
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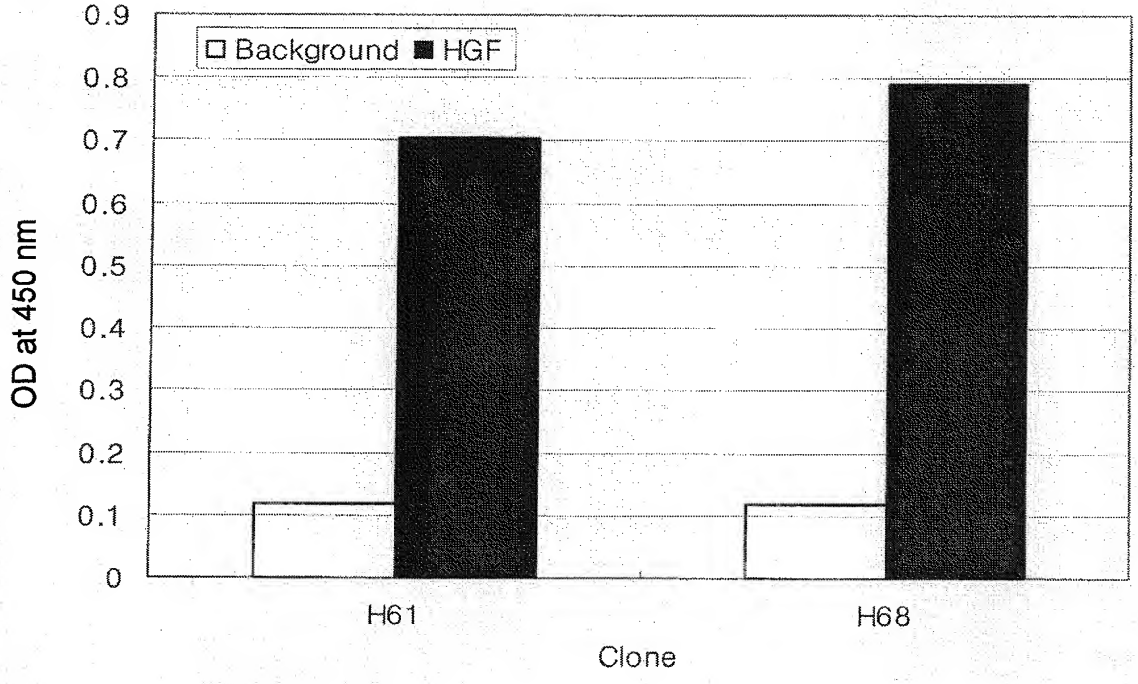
Anti HGF 68 Fab

Fig. 7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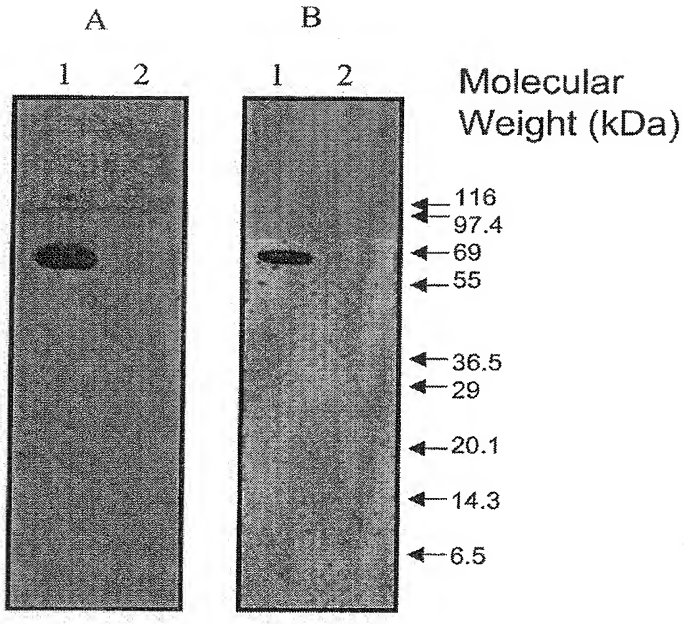
Clone

Fig. 8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6.5

Fig. 9A

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25	3	4	5	5	5	4
50	4	5	6	6	5	3
100	5	6	6	4	4	2
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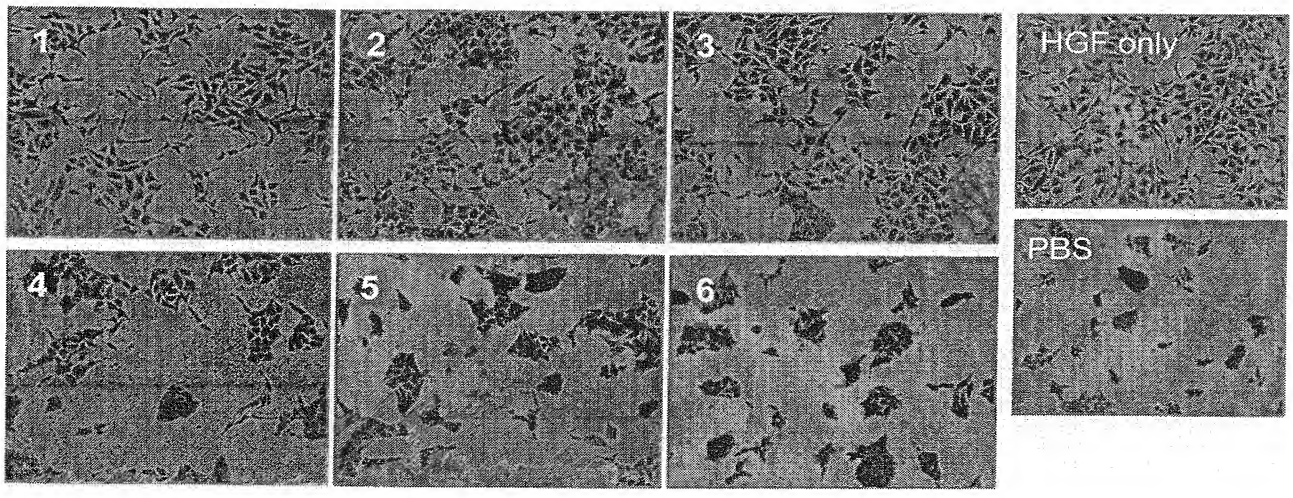
Fig. 9B



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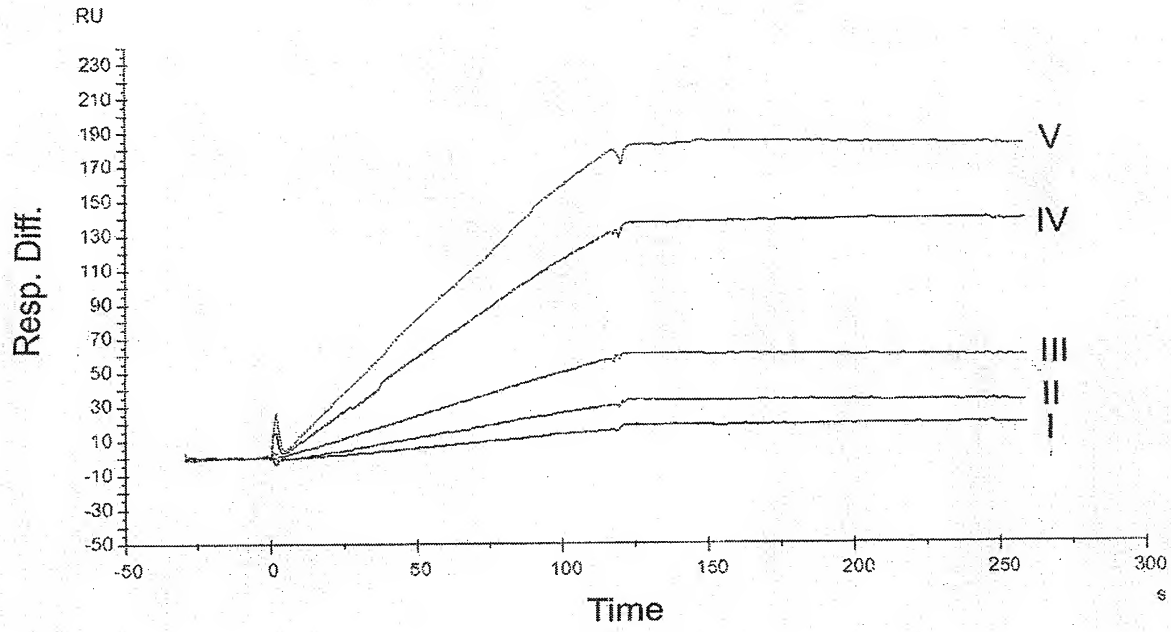
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Fig. 11

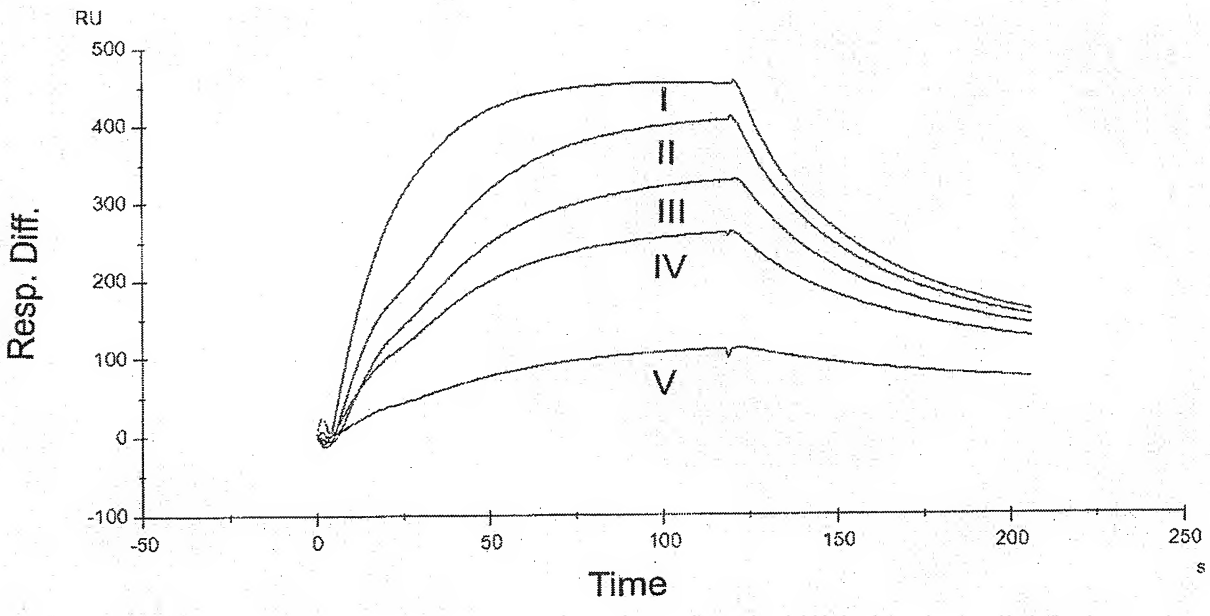
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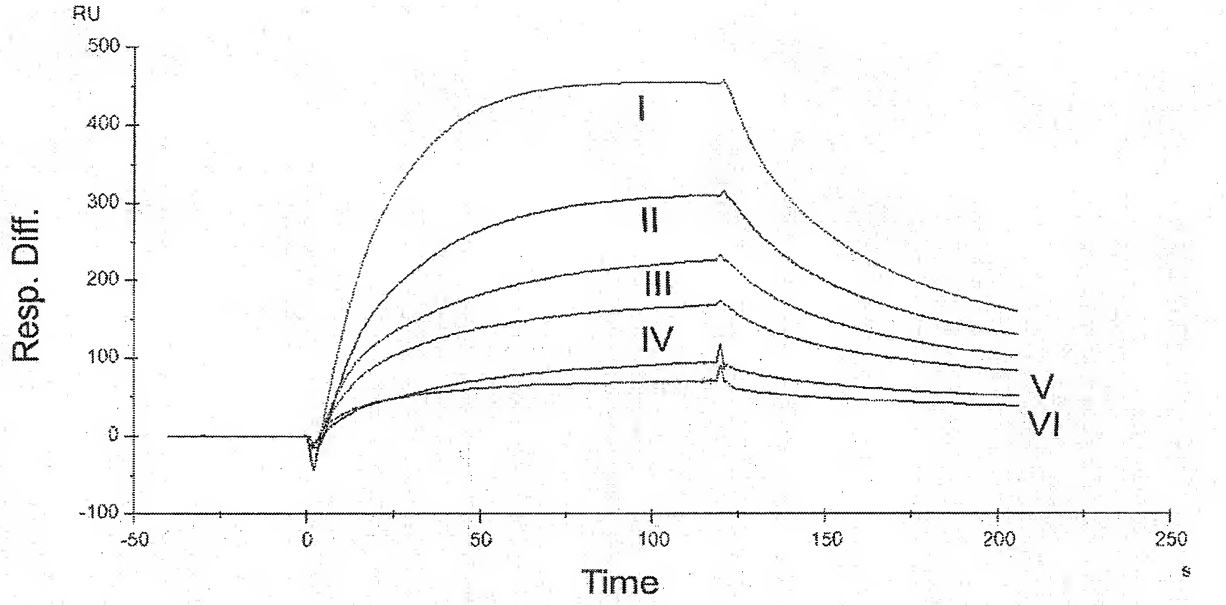
Fig. 12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gagctcgatc tgacccagac tccatcctct gtgtctgcag ctgtgggagg cacagtcacc 60

atcaattgcc aggccagtca gagtgttagc aacctcttag cctggtatca gcagaaacca 120

gggcagcctc ccaagctcct gatttatggt gcatccaatc tggaatctgg ggtcccatcg 180

cgtttccgtg gcagtggatc tgggacagag ttcactctca ccatcagtgg catgaaggct 240

gaagatgctg ccacttatta ctgtcaaagt ggttattata gtgctggtgc gacttttgga 300

gctggcacca atgtggaaat caaacga 327

<210> 27
<211> 116
<212> PRT
<213> Artificial Sequence

<220>
<223> amino acid sequence of VH region of clone 61

<400> 27
Gln Glu Gln Leu Met Glu Ser Gly Gly Arg Leu Val Asn Pro Gly Glu
1 5 10 15

Ser Leu Thr Leu Thr Cys Lys Ala Ser Gly Phe Thr Phe Ser Ser Tyr
20 25 30

Tyr Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
35 40 45

Gly Tyr Ile Gly Thr Ser Ser Gly Thr Thr Tyr Tyr Ala Asn Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Ser Asp Asn Ala Gln Asn Thr Val Phe
65 70 75 80

Leu Arg Met Thr Ser Leu Thr Asp Ser Asp Thr Ala Thr Tyr Phe Cys
85 90 95

Ala Arg Gly Leu Gly Arg Ile Asn Leu Trp Gly Pro Gly Thr Leu Val
100 105 110

Thr Val Ser Ser
115

<210> 28
<211> 109
<212> PRT
<213> Artificial Sequence

<220>
<223> amino acid sequence of VL region of clone 61

<400> 28
Glu Leu Val Leu Thr Gln Thr Pro Ser Ser Met Ser Ala Ala Val Gly
1 5 10 15

Gly Thr Val Thr Ile Asn Cys Gln Ala Ser Gln Ser Val Ser Asn Tyr
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile
35 40 45

Tyr Arg Ala Ser Thr Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Gly Met Lys Ala
65 70 75 80

Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Ser Gly Tyr Tyr Ser Ala Gly
85 90 95

Ala Thr Phe Gly Gly Gly Thr Asn Val Glu Ile Lys Arg
100 105

<210> 29
<211> 116
<212> PRT
<213> Artificial Sequence

<220>
<223> amino acid sequence of VH region of clone 68

<400> 29
Gln Gln Gln Leu Val Glu Ser Gly Gly Arg Leu Val Asn Pro Gly Glu
1 5 10 15

Ser Leu Thr Leu Thr Cys Lys Ala Ser Gly Phe Thr Phe Ser Thr Tyr
20 25 30

Tyr Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
35 40 45

Gly Tyr Ile Gly Thr Ser Ser Gly Thr Thr Tyr Tyr Ala Asn Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Ser Asp Asn Ala Gln Asn Thr Val Phe
65 70 75 80

Leu Gln Met Thr Ser Leu Thr Asp Ser Asp Thr Ala Thr Tyr Phe Cys
85 90 95

Ala Arg Gly Leu Gly Arg Ile Asn Leu Trp Gly Pro Gly Thr Leu Val
100 105 110

Thr Val Ser Ser
115

<210> 30
<211> 109
<212> PRT
<213> Artificial Sequence

<220>
<223> amino acid sequence of VL region of clone 68

<400> 30
Glu Leu Asp Leu Thr Gln Thr Pro Ser Ser Val Ser Ala Ala Val Gly
1 5 10 15

Gly Thr Val Thr Ile Asn Cys Gln Ala Ser Gln Ser Val Ser Asn Leu
20 25 30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile
35 40 45

Tyr Gly Ala Ser Asn Leu Glu Ser Gly Val Pro Ser Arg Phe Arg Gly
50 55 60

Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Gly Met Lys Ala
65 70 75 80

Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Ser Gly Tyr Tyr Ser Ala Gly
85 90 95

Ala Thr Phe Gly Ala Gly Thr Asn Val Glu Ile Lys Arg
100 105

<210> 31

<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> sequencing primer

<400> 31
ccctcatagt tagcgtaacg 20

<210> 32
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> neutralizable epitope of HGF

<400> 32
His His Pro His Phe Lys Pro Val Ser Asn Ser Arg
1 5 10

<210> 33
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> neutralizable epitope of HGF

<400> 33
Lys Ser Leu Ser Arg His Asp His Ile His His His
1 5 10

<210> 34

<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> nucleotide sequence encoding SEQ. ID. No. 32

<400> 34
catcatccgc attttaagcc tgtgtctaat agtcgt 36

<210> 35
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> nucleotide sequence encoding SEQ. ID. No. 33

<400> 35
aagtctctta gtcggcatga tcatattcat catcat 36